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Hepatitis Delta Virus Genotype IIb Predominates in an Endemic Area, Okinawa, Japan

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Hepatitis delta virus (HDV) infection is relatively common in the Miyako Islands, Okinawa, Japan, where the infection has been reported to be associated with low pathogenicity. HDV RNA extracted from each of 6 patients with HDV-related chronic liver disease living in the islands was amplified by reverse transcription-polymerase chain reaction and examined genetically to determine the HDV genotype. All isolates from the 6 patients were classified as genotype II by the neighbor-joining method. However, these isolates had relatively low homology (75–81%) to the HDV genotype II isolate reported from Japan, and showed relatively high identity (83–95%) to the novel genotype II isolate (HDV genotype IIb) recently reported from Taiwan. Phylogenetic analysis showed that the 6 isolates form a novel group within HDV genotype II. Furthermore, there was notable variation in sequence among the 6 isolates compared with the relatively close clustering of HDV isolates within limited areas (e.g., United States, Archangelos, Turkey, Albania, Peru). HDV genotype II in the Miyako Islands is therefore unique, and HDV infection may have been introduced at a relatively early time in this area. ***J. Med. Virol. 58:366–372, 1999.***

KEY WORDS: hepatitis delta virus; genotype; molecular epidemiology

INTRODUCTION

Hepatitis delta virus (HDV) is a single-stranded RNA virus that requires helper function of hepatitis B virus (HBV) [Rizzeto, 1983]. Many isolates of HDV have been reported from various areas [Wang et al., 1986; Makino et al., 1987; Chao et al., 1990; Imazeki et al., 1990, 1991; Lee et al., 1992; Casey et al., 1993; Wu et al., 1995; Lee et al., 1996; Shakil et al., 1997; Wu et al., 1998], and these isolates have so far been divided into 3 major genotypes (HDV genotypes I, II, III) [Casey et al., 1993]. HDV genotype I has been found worldwide, including in Japan [Wang et al., 1986; Makino et al., 1987; Chao et al., 1990; Imazeki et al., 1990; Lee et al., 1992; Casey et al., 1993; Shakil et al., 1997]. In contrast, HDV genotypes II and III are distributed in limited areas: genotype II in the Far East (Japan and Taiwan) [Imazeki et al., 1991; Wu et al., 1995; Lee et al., 1996; Wu et al., 1998] and genotype III in northern South America [Casey et al. 1993]. It is well known that HDV infection contributes to a severe form of acute hepatitis or chronic hepatitis, but the severity of liver disease varies according to the area [Polish et al., 1993]. HDV infection in northern South America is associated frequently with fulminant hepatitis [Polish et al., 1993; Casey et al., 1996]. In contrast, the disease appears to be mild in some areas, such as the community of Archangelos on the Greek island of Rhodes [Hadziyannis et al., 1987], Pacific Islands [Hadler et al., 1991], and the Far East [Wu et al., 1995]. The reason for this variation is not known, but it may be related to differences in the strains of HDV.

Recently, the existence was reported of an endemic focus of HDV infection in the Miyako Islands group, Okinawa, Japan, where HDV infection is associated with low pathogenicity [Sakugawa et al., 1997a]. As the HDV genotype in the islands has not yet been characterized, a molecular-epidemiological study was undertaken to determine the genotype.

The nucleotide sequence data reported in this article will appear in the DDBJ/EMBL/GenBank nucleotide sequence databases with accession numbers *ABO 15442–ABO 15447.*

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TABLE I. Clinical Data and Large:Small (L:S) Genome Ratio of the 6 Patients Analyzed

Patient	Age (years)	Sex	Birth place	Diagnosis	ALT[a]	No. of clones	L-genome (%)
1	36	M	Hirara City	CH	77	12	33
2	74	M	Hirara City	HCC + LC	27	11	18
3	63	F	Hirara City	HCC	45	12	100
4	57	F	Hirara City	LC	36	12	8
5	48	F	Hirara City	CH	42	12	50
6	66	F	Irabu Island	LC	24	12	25

[a]ALT, alanine aminotransferase.

MATERIALS AND METHODS

Patient Population

Twenty-six patients with HDV-related chronic liver disease associated with chronic HDV infection who consecutively visited the Okinawa Prefectural Miyako Hospital were examined for HDV RNA by reverse transcription-polymerase chain reaction (RT-PCR), the method described previously [Sakugawa et al., 1997b]. The 26 patients consisted of 10 patients with chronic hepatitis (CH), 10 patients with liver cirrhosis (LC), and 6 patients with hepatocellular carcinoma (HCC). All 26 had detectable levels of HDV RNA in their serum: 13 showed strong reactivity (++) and 13 showed weak reactivity (+) (the method of judging the reaction strength was previously reported [Sakugawa et al., 1997b]). To maximize the amount of viral template, serum samples were collected from the 13 patients with strong reactivity by RT-PCR for sequencing the HDV genomes. The 13 patients consisted of 5 patients with CH, 4 patients with LC, and 4 patients with HCC. A total of 6 sera, which were composed of 2 randomly selected sera from each of the 3 patient groups (CH, LC, HCC), were selected for nucleotide sequence analysis. The 6 patients thus examined consisted of 2 males and 4 females, aged 36–74 years. All 6 patients resided in the Miyako Islands group, 5 in Hirara City in the main Miyako Island, and one in the Irabu Island (Table I). None of the patients was related directly to each other, nor did they inhabit the same residence. The clinical history of these patients was characterized as generally asymptomatic with slowly progressive liver disease. All 6 were negative for hepatitis Be (HBe) antigen and positive for antibody to HBe, and had negative reaction for antibody to hepatitis C virus (HCV). None of the 6 patients had a history of blood transfusion, or was included in a high-risk group for parenterally transmitted disease (i.e., intravenous drug abusers, prostitutes, homosexuals).

RT-PCR

HDV RNA in patients' serum was reverse transcribed with the external antisense primer (HDV-R1, Table II). RT was performed at 37°C for 60 min in a mixture (final volume 20 μl) containing RNA template, 10 mM dithiothreitol, 4 μl each of deoxyribonucleoside triphosphate and 100 pmol of the primer, ribonuclease inhibitor (Gibco-BRL, Gaithersburg, MD), and 108 units of reverse transcriptase from Moloney murine leukemia virus (Gibco-BRL).

PCR amplification was carried out by adding 100 μl of 1 × XL buffer containing 100 pmol of the external sense primer (HDV-F1, Table II) and 4 units of rTth DNA polymerase (Perkin-Elmer, Foster City, CA). The mixture was overlaid with mineral oil. A Robo Cycler™ PCR machine (Stratagene, Los Angeles, CA) was programmed to incubate samples for 30 cycles at 94°C for 30 sec, 45°C for 1.5 min, and 72°C for 3.5 min, followed by a 10 min final extension at 72°C.

For the second amplification, a 5 μl aliquot of the resulting mixture in the first PCR was added to a reaction mixture similar to the first reaction but with 100 pmol of the internal primers (HDV-F2 and HDV-R2, Table II) instead of the external primers. PCR was carried out as described for the first amplification.

Sequence Analysis

The PCR-amplified fragments were cloned into the TA cloning site of pCR II™ cloning vector (Invitrogen Co., Carlsbad, CA) using T4 DNA Ligase (TaKaRa Shuzo, Shiga, Japan). Sequence analysis of these PCR-amplified fragments in the cloning vector was conducted using an ABIPRISM™ 377 DNA sequencer (Perkin-Elmer) with M13 sequencing primers.

Phylogenetic analysis was carried out using the 6-parameter, neighbor-joining method, as described previously [Doi et al., 1996].

RESULTS

The nucleotide sequences obtained in this study covered nucleotides (nt) 940–1584 (numbering according to Wang et al. [1986]). This region (nt 940–1584) begins near the polyadenylation signal of the viral mRNA and includes the RNA editing site and the sequences encoding the viral protein (except for 5 amino acids of its N-terminus).

Eleven or 12 clones from each subject were sequenced, and the mean heterogeneity among different clones of a single subject ranged from 1.18% to 4.43%. All subjects had the genome coding for the large form of delta antigen (L-genome). The ratio of small:large (S:L) genomes varied among these subjects and did not correlate with the disease activity or severity (Table I). The most representative clones were selected from each of the 6 subjects, and their nucleotide sequences

TABLE II. HDV Primers Used in This Study

Primers	Sequences	Location[a]
Sense		
HDV-F1	5′-CCTACGGATGCCCAGGTCGGACCGCGAGGAGGTGGAGATG-3′	854–893
HDV-F2	5′-CCCGAAGAGGAAAGAAGAACTCGGACGCGAACCGTGAGTG-3′	903–942
Antisense		
HDV-R1	5′-TTTCAAAAAGAAAGAATAGAGAGAACTGAGGACCCTCGCC-3′	1611–1650
HDV-R2	5′-AACTGAGGACCCTCGCCCGACCTGAGAATGAGCCAATCCG-3′	1588–1627

[a]Numbering according to Imazeki et al. [1991].

were compared with each other or with those of other isolates of HDV reported previously from other areas of the world. The HDV isolates derived from the patients living in the Miyako Islands are 83.8–96.1% (median sequence homology: 91.4%) identical to one another. Comparisons of these 6 sequences with published sequences of isolates representative of the 3 genotypes of HDV (Italy, Japan-1, and Peru-1) over the nt 940–1584 region indicated that the isolates obtained in this study were distantly related to characterized isolates: 71.0–77.5%, 75.3–80.9%, and 63.0–68.3% homology to genotype I (Italy), II (Japan-1), and III (Peru-1), respectively (Figs. 1, 2). However, these isolates from the Miyako Islands showed a relatively high identity to the isolate (TWD62) recently reported from Taiwan [Wu et al., 1998]. Japan-1 and TWD62 are here designated "IIa" and "IIb," respectively, according to Wu et al. [1998] (Fig. 1). There are marked variations in divergence in different regions of the HDV sequence (nt 940–1584) among these 6 isolates and the different genotypes. A great divergence was seen at both ends of the sequences, which corresponded to the C- or N-end of the large delta antigen (Fig. 1). In these divergent regions, our isolates showed a higher identity to genotype II than to genotype I or III. When compared with the prototype sequence (genotype I), there is a mutation at position 1014 (T $\rightarrow$ C) in every sequence (and every single clone) obtained in this study. In the middle part of delta antigen coding region, there are some well-conserved regions (nt 1156–1225, nt 1254–1298, nt 1334–1402) among the different genotypes. These regions corresponded to the RNA-binding domain (nt 1156–1225 and nt 1254–1298) or the nuclear localization signal region (nt 1334–1402) [Lai, 1995]. The 6 isolates obtained in this study had a higher identity to the prototype sequence than to genotype II in the region from nt 1156 to 1225.

Phylogenetic analysis of sequences over the nt 940–1584 region by the neighbor-joining method indicates that all isolates obtained in this study are classified into HDV genotype II. However, these isolates from the Miyako Islands and TWD62 [Wu et al., 1998] form a subgroup within HDV genotype II (HDV genotype IIb). Japan-1 and Taiwan-3 are 92.7% identical to one another (genotype IIa) (Fig. 2). The nucleotide sequences obtained in this study covered most of the region (nt 911–1260) used for the classification of HDV genotypes by Casey et al. [1993] (our sequences do not cover only a 29-nucleotide length of the 5′-end of the region proposed for genotyping). The phylogram obtained from the comparison of isolates over the 940–1260 region was similar to that obtained from the comparison of the nt 940–1584 region (data not shown).

Comparison of the nucleotide sequences obtained in this study with each other indicates that the 2-05 isolate (Fig. 1) is the most representative one among the 6 isolates. The predicted amino acid sequence of HDAg for the 2-05 isolate is compared with the isolates Italy (genotype I), Japan-1 (genotype IIa), TWD62 (genotype IIb), and Peur-1 (genotype III) in Figure 3. In this comparison, 2-05 is about 87% identical to TWD62, and is about 75%, 69%, and 58% identical to Japan-1, Italy, and Peur-1, respectively. Thus, at both the amino acid and nucleic acid levels, HDV isolates in the Miyako Islands, as represented by isolate 2-05, are more distantly related to the genotype II HDV isolate reported previously from Japan. There are great variations in identity in various domains among 2-05 and the different genotypes. Both the RNA-binding domain and the nuclear localization signal region [Lai, 1995] were well conserved. In contrast, the N-terminus and the C-terminal packaging sequence were less conserved. Of note, the HDV genotype IIb (TW and 2-05) showed a higher identity with genotype I in these well-conserved regions, but a higher identity with genotype IIa in these less-conserved areas.

DISCUSSION

Clinical studies of HDV disease worldwide indicate that there is a wide variation in pathogenesis [Hadzyannis et al., 1987; Hadler et al., 1991; Polish et al., 1993; Wu et al., 1995; Casey et al., 1996], and the reasons for these differences are presently unknown. It has been suggested that difference in severity of HDV-related liver disease is attributable to heterogeneity of the HDV genome [Casey et al., 1993; Wu et al., 1995]. A vast majority of published HDV isolates belong to genotype I [Wang et al., 1986; Makino et al., 1987; Chao et al., 1990; Imazeki et al., 1990; Lee et al., 1992; Casey et al., 1993; Shakil et al., 1997]. Since these isolates were derived from many diverse geographical areas and were associated with various clinical pictures, the genotype I HDV may be a very heterogeneous group. In contrast, genotype III isolates were restricted so far to northern South America [Casey et al., 1993] and were associated with outbreaks of a severe form of acute hepatitis [Casey et al., 1993, 1996]. Genotype II isolates were reported only from the Far East (Japan and Taiwan) [Imazeki et al., 1991; Wu et al., 1995; Lee

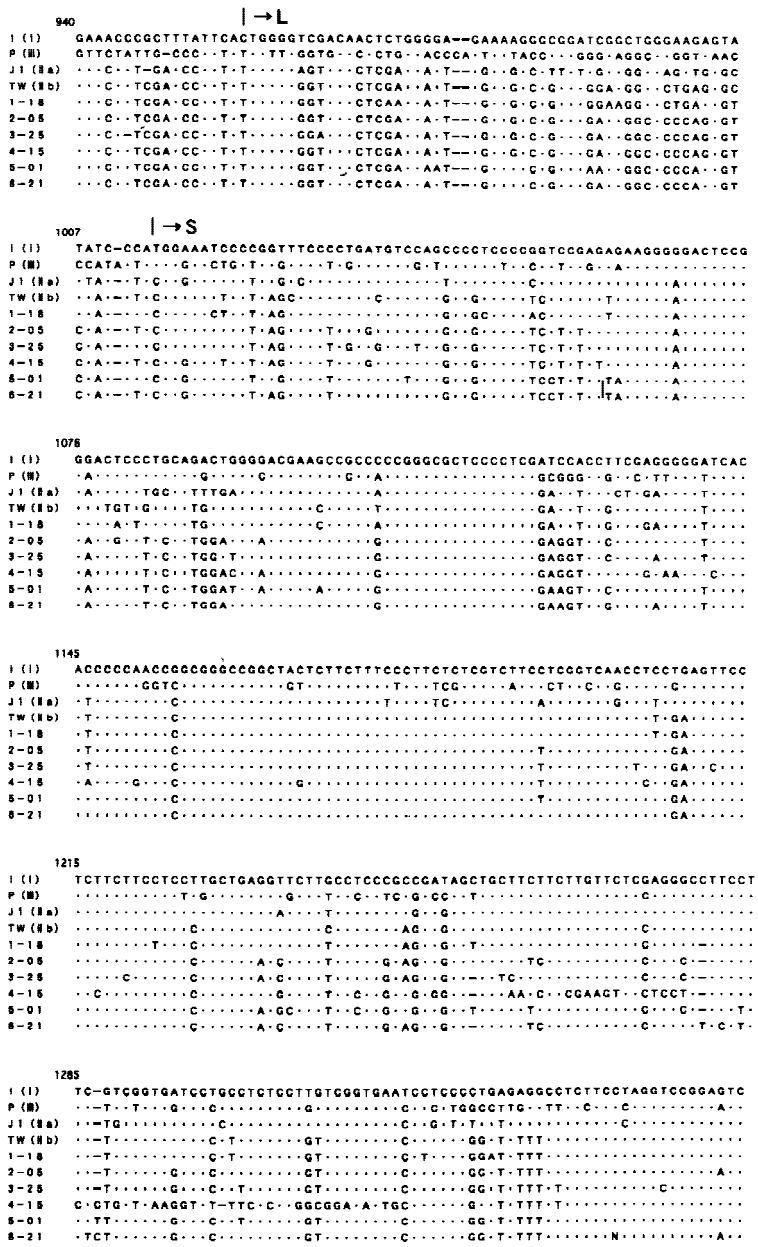

Fig. 1. Comparison of genomic sequences (nt 940–1584) of HDV isolates obtained in this study with published HDV sequences. The numbering of nucleotides is according to Wang et al. [1986]. The vertical lines indicate the termination sites for the small HD antigen (S) and the large HD antigen (L), respectively. Dots indicate conserved nucleotides and dashes indicate missing nucleotides. Isolates 1-18, 2-05, 3-25, 4-15, 5-01, and 6-21 are from this study (obtained from patients 1, 2, 3, 4, 5, and 6 [see Table I], respectively). Sources of other isolates are as indicated: Italy (I, genotype I) [Wang et al., 1986], Peru (P, genotype III) [Casey et al., 1993], Japan-1 (J1, genotype IIa) [Imazeki et al., 1991], and TWD62 (TW, genotype IIb) [Wu et al., 1998].

et al., 1996; Wu et al., 1998], where HDV infection is relatively infrequent and is known to be associated with low pathogenicity [Wu et al., 1995]. The genetic heterogeneity of HDV isolates may also account for at least part of the difference in the clinical picture of HDV infection.

The Miyako Islands are known to be an endemic area of both HBV and HDV infection [Sakugawa et al., 1997a]. The majority of patients with chronic HDV-related liver disease in the Miyako Islands experience a relatively benign, asymptomatic course that evolves to a quiescent cirrhosis condition (our unpublished observation). All of the 6 subjects in this study had stable chronic liver disease showing a serum alanine aminotransferase level in the normal or nearly normal range (less than twice the upper limit of the normal range). Furthermore, HDV isolates obtained from these patients belong to genotype II, which is thought to be associated with low pathogenicity [Wu et al., 1995]. The relation between HDV genotype II infection and the clinical picture of mild hepatitis has not yet been established, because there have so far been very few

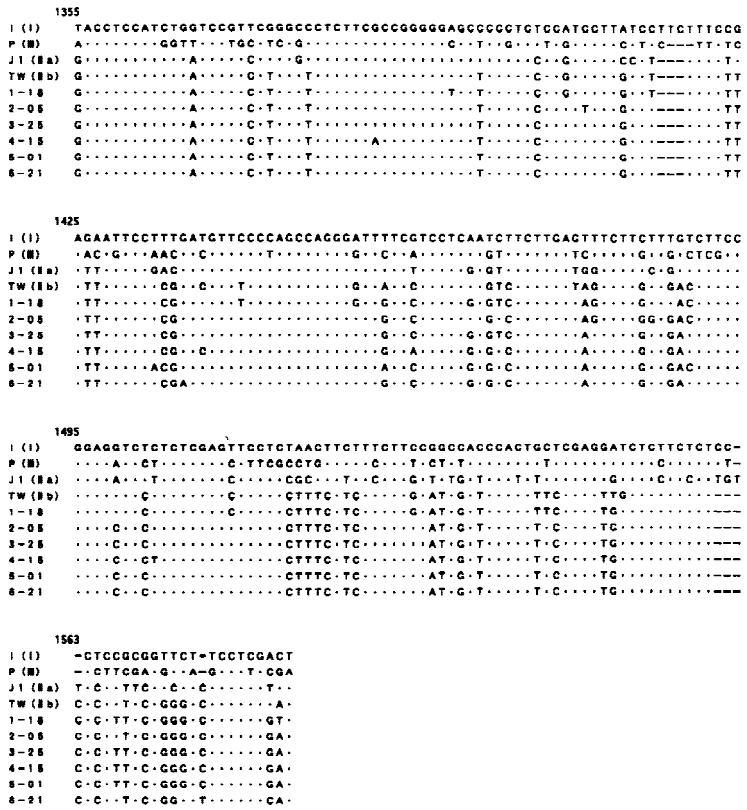

Fig. 1. Continued.

reported patients infected with genotype II HDV. Moreover, the patients reported had established chronic HDV infection, which is known to be less severe than the acute phase. In this study, we have also analyzed sera obtained from chronically infected patients. Further studies are needed to clarify whether HDV genotype II infection is indeed associated with low pathogenicity.

There are 2 forms of hepatitis delta antigen (HDAg): small HDAg and large HDAg. Production of the 2 forms of HDAg is regulated by an RNA editing mechanism: a base change (A $\rightarrow$ G) at position 1012 in the antigenomic RNA of HDV [Casey et al., 1995]. The genome coding for the small HDAg is replication competent, whereas the genome coding for the large HDAg is nonreplicating. The possibility that the ratio of S:L genomes may affect the pathogenicity of HDV infection has been advanced [Govindarajan et al., 1993; Tang et al., 1994]. In this study, the S:L genome ratio did not correlate with disease severity. The RNA editing of position 1012 is critically dependent on the sequence of the surrounding nucleotides [Casey et al., 1992]. All isolates obtained in this study had a nucleotide change at position 1014 (T $\rightarrow$ C), and this nucleotide substitution is constantly seen in all HDV genotype II isolates published. The mutation is also seen in some HDV genotype I isolates, e.g., Nauru [Chao et al., 1990] and Greek [Yang et al., 1995] isolates. Of interest is that these HDV genotype I isolates with the mutation (1014 C) are associated with less severe liver disease [Hadler et al., 1991; Yang et al., 1995].

Although the HDV isolates obtained in this study are classified as genotype II by phylogenetic analysis, there are significant differences between these isolates and the published isolates of HDV genotype II; Japan-1 and Taiwan-3 are closely related to one another (about 93% homology) despite geographical distance. The HDV isolates obtained in this study could be phylogenetically classified as a new genotype subgroup within HDV genotype II. Recently, Wu et al. [1998] reported 5 HDV isolates that formed a novel subgroup within HDV genotype II and classified these isolates as "IIb." One of the nucleotide sequence data of the 5 isolates is now available (GenBank accession no. AF018077), and the isolate is found to be similar to the isolates obtained in this study. Phylogenetic analysis showed that all our isolates are classified as HDV genotype IIb.

The variation of HDV sequences in the Miyako Islands within genotype II was particularly notable when compared with the relatively low diversity of the sequence of genotype IIa isolates previously reported from Japan and Taiwan [Imazeki et al., 1991; Lee et al., 1996]. The degree of sequence diversity of HDV isolates within geographic regions is variable. Median sequence diversity was reported to be low (3.1–5.3%) among isolates from the United States, Archangelos, Turkey, Albania, and Peru, whereas high sequence diversity (8.0–13.4%) was seen in Italy, mainland Greece,

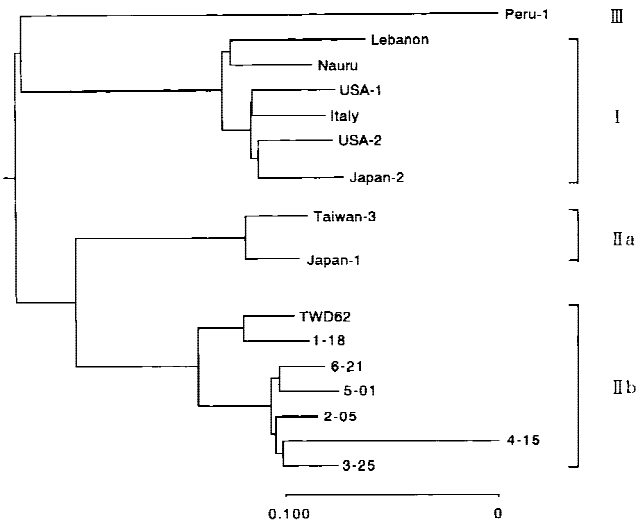

Fig. 2. Phylogram generated by neighbor-joining analysis showing relation of HDV isolates to one another. Nucleotides 940–1584 (numbering according to Wang et al. [1986]) from different isolates were compared. Isolates 1-18, 2-05, 3-25, 4-15, 5-01, and 6-21 are from this study. Sources of other isolates are as indicated: Italy [Wang et al., 1986], USA-1 [Makino et al., 1987], USA-2 [Casey et al., 1993], Lebanon [Lee et al., 1992], Nauru [Chao et al., 1990], Japan-2 [Imazeki et al., 1990], Japan-1 [Imazeki et al., 1991], Taiwan-3 [Lee et al., 1996], TWD62 [Wu et al., 1998], and Peru-1 [Casey et al., 1993].

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I (I)        6  SRKNR-GGREEILEQWVAGRKKLEELERDLRKTKKKLKKIEDENPW
P (III)         A·LTS-KE·········EE··NRRK··K···RAN··I··L·····
J-1 (IIa)       T·RG·R·T···T··K·ITA···A····K·····R·TI··L·E····
TW (IIb)        ··RP·R·=···Q·GK·IDA·RRK·········VN·TI·RL·ED···
2-05            P·RP·R·=···T·GK·ID··RRK·····E···VT·T··RL·ED···

I (I)       51  LGNIKGILGKKDKDGEGAPPAKRARTDQMEVDSGPRKRPLRGGFTDKERQ
P (III)         ···VV·L·=RRK··ED·····P·QET·····GRK·KAR····Q··R
J-1 (IIa)       ····V··IR·=G··········P··········G···HKS·····E
TW (IIb)        ···VR··IR·=·····························KHP····EQ··R
2-05            ····R··IR·=···N·························KHP····EQ··R

I (I)      101  DHRRRKALENKKKQLSAGGKNLSKEEEEELRRLTEEDERRERRVAGPPVG
P (III)         ··············AG···H··Q·········ARD·DE····T···RP·
J-1 (IIa)       ····················I·············D···E·K·····R··
TW (IIb)        ·················S·····R·····················R··
2-05            ·····=G····R····S···SF·R··········K··········R··

I (I)      151  GVIPLEGGSRGAPGGGFVPSLQGVPESPFSRTGEGLDIRGNRGFPWDILF
P (III)         ··N·MD·PP·····························I····TQQ···YGFT
J-1 (IIa)       D·N·SR··P··········QMA·················TQ····VSPS
TW (IIb)        D·N··D··P··········MHDI·····T·R·D···V··AQE···VSPQ
2-05            D·N····PP··········ME·········R·D···T··TQ····VDPG
                                                          ★

I (I)      201  PADPP-FSPQSCRPQ
P (III)         ·PP·GYYWVPG·TQ·
J-1 (IIa)       ·PQQR-LPLLE·T··
TW (IIb)        ·PP·R-LPLLE·T··
2-05            RPS·R-LPLLE·T··
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Fig. 3. Comparison of the amino acid sequence of HDAg between 2-05 (representative one among 6 isolates obtained in this study) and published sequences: I (Italy, genotype I), P (Peru-1, genotype III), J-1 (Japan-1, genotype IIa), and TW (TWD62, genotype IIb). Our isolate does not cover the 5-amino-acid length of its N-terminus, so that the region (amino acids 6–214) is compared. A star indicates the C-terminal amino acid in small HDAg.

and north Africa [Casey et al., 1996; Shakil et al., 1997]. It was suggested that HDV was introduced more recently and/or from fewer sources into the geographic areas with low sequence diversity compared with the regions with high sequence diversity [Shakil et al., 1997]. The mean sequence diversity among the 6 isolates obtained in the Miyako Islands was 8.6%; the figure is comparable to those seen in Italy and mainland Greece. On the contrary, the 5 HDV genotype IIb isolates reported from Taiwan showed >97% identity with each other [Wu et al., 1998]. The Miyako Islands are small, isolated islands; most inhabitants in the islands are Japanese and originate from the islands. All of the subjects in this study are of Miyako origin, and none of them is an intravenous drug abuser or belongs to any other high-risk group for HDV infection. The HDV isolates obtained in this study may reflect a genuine geographical variation rather than an imported infection of the virus. These data suggested that HDV genotype II has been introduced much earlier in the Miyako Islands than in the other areas of east Asia, and that HDV infection has persisted in the islands for a relatively long time without introduction of other genotypes of HDV.

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